Am J Physiol Cell Physiol 290: C1583–C1591, 2006.
First published January 25, 2006; doi:10.1152/ajpcell.00390.2005.

PKC-δ mediates activation of ERK1/2 and induction of iNOS by IL-1β in vascular smooth muscle cells

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Submitted 2 August 2005; accepted in final form 19 January 2006

Ginnan, Roman, Benjamin J. Guikema, Harold A. Singer, and David Jourd'heuil. PKC-δ mediates activation of ERK1/2 and induction of iNOS by IL-1β in vascular smooth muscle cells. *Am J Physiol Cell Physiol* 290: C1583–C1591, 2006. First published January 25, 2006; doi:10.1152/ajpcell.00390.2005.—Although the inflammatory cytokine interleukin-1β (IL-β) is an important regulator of gene expression in vascular smooth muscle (VSM), the signal transduction pathways leading to transcriptional activation upon IL-1β stimulation are poorly understood. Recent studies have implicated IL-1β-mediated ERK1/2 activation in the upregulation of type II nitric oxide synthase (iNOS) in VSM. We report that these events are mediated in a phospholipase C (PLC)- and protein kinase C (PKC)-δ-dependent manner utilizing a signaling mechanism independent of $p21^{ras}$ (Ras) and Raf1 activation. Stimulation of rat aortic VSM cells with IL-1β activated PLC-γ and pharmacological inhibition of PLC attenuated IL-1β-induced ERK1/2 activation and subsequent iNOS expression. Stimulation with IL-1β activated PKC-α and -δ, which was blocked using the PLC inhibitor U-73122. Pharmacological studies using isoform-specific PKC inhibitors and adenoviral overexpression of constitutively active PKC-δ indicated that ERK1/2 activation was PKC-α independent and PKC-δ dependent. Similarly, adenoviral overexpression of constitutively activated PKC-δ enhanced iNOS expression. IL-1β stimulation did not induce either Ras or Raf1 activity. The absence of a functional role for Ras and Raf1 related to ERK1/2 activation and iNOS expression was further confirmed by adenoviral overexpression of dominant-negative Ras and treatment with the Raf1 inhibitor GW5074. Taken together, we have outlined a novel transduction pathway implicating PKC-δ as a critical component of the IL-1-dependent activation of ERK in VSM cells.

nitric oxide synthase

THE RESPONSE of the vessel wall to injury and inflammation is most often associated with leukocyte infiltration and a coordinated increase in growth factors and cytokines including basic fibroblast growth factor, PDGF, tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β). This culminates ultimately in an increased proliferative activity of vascular smooth muscle cells and the formation of neointima, which is of central importance in the development of vascular diseases such as restenosis and atherosclerosis (24, 35).

IL-1β is a multifunctional cytokine that is produced by a variety of cells in the vascular wall, including macrophages, fibroblasts, and endothelial and smooth muscle cells (5, 7). IL-1β actively participates in the regulation of smooth muscle cell function by modulating gene expression and proliferation (3). The signaling cascade initiated by IL-1β has been characterized to some extent and has been shown to result in the activation of proinflammatory transcription factors, including AP-1 and NF-κB (1). This involves the successive recruitment and/or activation of IL-1 receptor-associated kinase (IRAK) (28), SHP2, a tyrosine phosphatase (27), TRAF6 (15), and a succession of kinase enzymes, such as NF-κB-inducing kinase (29). More recently, the extracellular signal-regulated kinases (ERKs) have been shown to be essential for the persistent activation of NF-κB and the consequential expression of proinflammatory genes including the inducible nitric oxide (NO) synthase (iNOS) (21, 22). We (18) recently reported that treatment of vascular smooth muscle (VSM) cells with IL-1β results in an ERK1/2-dependent regulation of iNOS expression that is negatively regulated by ROS and p38 MAPK. Although the distal events dealing with the regulation of NF-κB by ERKs are understood to some extent, it is evident that the signaling cascade leading to IL-1β-mediated activation of ERK1/2 itself is poorly understood. It has been reported that IL-1β-dependent activation of ERK1/2 may be focal adhesion dependent (28) involving SHP2 (27) and phospholipase C-γ (PLC-γ) (45). In addition, whereas activation of MEK1/2 is apparently required based on the ability of U0126, a selective MEK inhibitor, to attenuate ERK1/2 activation (18), the role of classic ERK1/2 signaling molecules, such as $p21^{ras}$ (Ras) and Raf, is not clear. There are no reports that describe the signaling intermediates necessary for IL-1β stimulation of ERK1/2 in VSM.

PKCs have been implicated in IL-1β-dependent signaling and iNOS expression, although their role is somewhat confounding due to the potentially opposing effects of multiple PKC isozymes in the same cell type, and conflicting data resulting from alternate methods of inhibiting PKC activity (30). Some studies have reported a positive role for PKCs in mediating IL-1β signaling. For example, PKC-δ (4) and PKC-ε (2) have been shown to mediate upregulation of iNOS expression in response to stimulation with IL-1β. Conversely, other studies (19, 37) have shown that treatment with PKC inhibitors resulted in the upregulation of iNOS, suggesting that PKC activity serves to suppress iNOS expression. The mechanisms by which PKCs may regulate IL-1β-induced iNOS expression are not completely clear. A recent study by Carpenter et al. (4) identified PKC-δ as important for stabilizing iNOS mRNA. Which PKC isozymes are involved in iNOS regulation and whether they play a positive or negative role in that regulation is most likely cell and tissue dependent and dependent on the complement of PKC isozymes present.

PKCs are a ubiquitously expressed family of proteins with 11 members subdivided into three groups based on their requirements for activation (33, 36, 41). The conventional

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PKCs (α, β, and βγ) require Ca^{2+}, and the lipid activators diacylglycerol (DAG) and phosphatidylserine (PS) as prerequisites for activation. The novel PKCs (δ, ϵ, θ, η, μ) require only DAG and PS to catalyze their activation. The third subfamily of PKC is the atypical PKCs (ζ, ι/λ). This class of PKCs requires neither Ca^{2+} nor DAG for activation. There may be a role for PS in activation of atypical PKCs, but the molecular mechanisms are not completely understood. The PKC isozymes predominantly expressed in our VSM cultures are PKC-α and PKC-δ, although PKC-ϵ and PKC-ζ isoenzymes are also present (11, 41).

Given the importance of IL-1β signaling as it relates to the pathophysiology of vascular disease, the purpose of this study was twofold. First, our goal was to better understand the mechanisms that govern IL-1β signaling in VSM cells. Second, given that we have recently identified a role for PKC-δ in PDGF- and ATP-dependent activation of ERK1/2 in VSM cells (11, 12), we wanted to determine whether PKCs may mediate IL-1β-induced activation of ERK1/2 and subsequent iNOS expression and identify the specific isoform that is involved. Our data indicate that IL-1β-dependent ERK1/2 signaling in VSM cells involves PLC and utilizes the PKC isoform PKC-δ. Interestingly, this ERK1/2 signaling pathway appears to be Ras and Raf-1 independent.

EXPERIMENTAL PROCEDURES

Cell culture. VSM cells were obtained from the medial layer of the thoracic aorta of 200–300 g wt Sprague-Dawley rats as described by Geisterfer et al. (10). After the adventitial and endothelial layers were removed, medial smooth muscle cells were enzymatically dispersed and cultured in DMEM/F12 + 10% fetal bovine serum (Hyclone). The VSM cells were maintained at 37°C with 5% CO_2 and split twice a week. All experiments were performed on cells passaged 3–10 times. Before experimental use, subconfluent cultures (60–75%) were growth arrested for 16–24 h by exchanging the growth media with DMEM/F12 without serum. DMEM/F12 without serum was replaced with Hanks' balanced salt solution containing Mg^{2+} and Ca^{2+} and 10 mM HEPES (pH 7.4) for 30–60 min before treatment.

Cell lysates and Western blot analysis. Cells were lysed (0.5 ml/60 mm dish or 1 ml/100 mm dish) in a modified RIPA buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 10% glycerol, 1 mM DTT, 0.1 mM PMSF, and 0.2 U/ml aprotinin). Samples were resolved using standard SDS-PAGE procedures, transferred to nylon-backed nitrocellulose (MSI), and immunoblotted. After being blocked in 5% nonfat dry milk or 3% BSA, the immunoblots were incubated for either 1 h at room temperature or overnight at 4°C, washed 3 × 10 min with 20 mM Tris, 150 mM NaCl, and 0.2% Tween 20 (TBST) and incubated for 1 h with appropriate secondary antibody (HRP conjugate, Amersham). The blots were then washed 3 × 10 min with TBST, incubated in Enhanced Chemiluminescent substrate (Amersham) and exposed to X-ray film (Parker).

PKC activity assay. PKC-δ was immunoprecipitated from VSM cells and assayed as described earlier (26). After being washed three times with immunoprecipitation buffer and once in sucrose buffer (10 mM MOPS, pH 7.4, 250 mM sucrose, 2.5 mM EGTA, 2 mM EDTA, 0.2 U/ml aprotinin, and 0.2 mM PMSF), the protein A beads were incubated for 10 min at 30°C in 50 mM HEPES, pH 7.4, 10 mM $Mg(Ac)_2$, 2 mM $CaCl_2$, 1 mM EGTA, 0.2 mg/ml Histone IIIS, 1.4 μg/μl PS, 0.2 μg/μl diolein, 1 mM ATP, and 2 μCi/reaction [^{32}P]ATP. After incubation, 25 μl of reaction were spotted onto P81 filter paper, washed five times in 75 mM phosphoric acid, and once in ethanol. After drying, ^{32}P-incorporation was determined with the use of a scintillation counter (model LS6500; Beckman).

VSM cell fractionation and PKC translocation assay. After treatment, VSM cells were harvested and Dounce homogenized in a nondetergent sucrose buffer. The cell lysates were fractionated with a low-speed spin (1,000 *g*) to remove nuclei and intact cells. The supernatants were then centrifuged at 100,000 *g* to separate the "cytosolic" fraction from the particulate fraction. RIPA buffer was added to the particulate pellet to extract the "membrane" fraction from the insoluble particulate. The membrane fraction was resolved on SDS-PAGE and transblotted as described above. PKC activity was assessed as the amount of PKC present in the membrane fraction.

Ras activation assays. The activation state of Ras was determined using the Ras Activation Assay kit from Upstate Biotechnology. This

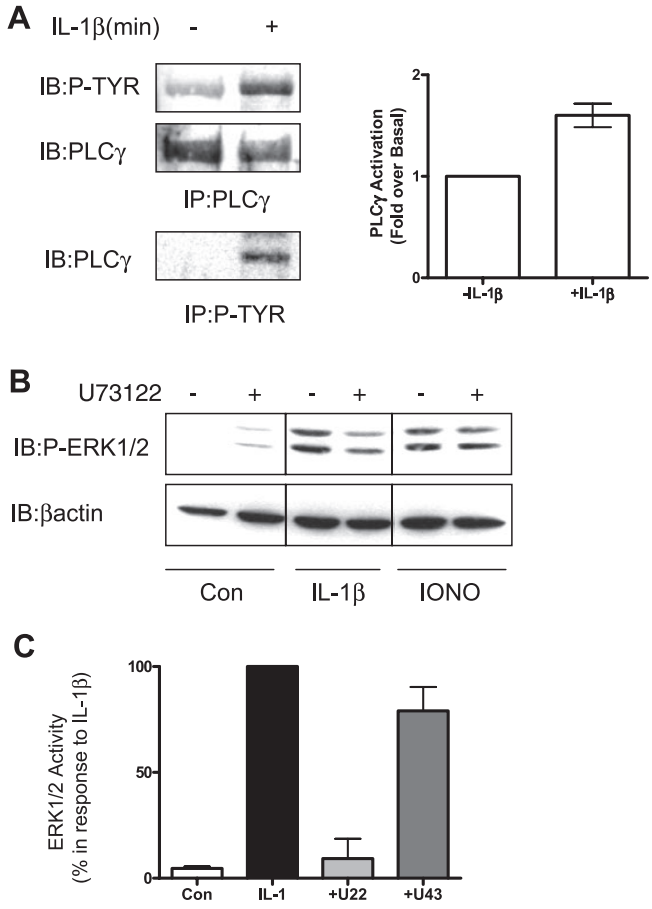

Fig. 1. IL-1β-dependent activation of phospholipase C-γ (PLC-γ) and ERK1/2. *A*: growth-arrested vascular smooth muscle (VSM) cells were treated with 10 ng/ml interleukin-1β (IL-1β) for 10 min. The cells were harvested in immunoprecipitation (IP) buffer and PLC-γ (IP:PLCγ) or tyrosine phosphorylated proteins (IP:P-TYR) were immunoprecipitated as described in EXPERIMENTAL PROCEDURES. The IP buffers were resolved on SDS-PAGE, transferred to nitrocellulose, and immunoblotted (IB) with antibody recognizing tyrosine-phosphorylated proteins (IB:P-TYR) or PLC-γ (IB:PLC-γ). The nitrocellulose membrane was stripped and immunoblotted with antibody recognizing PLC-γ (IB: PLC-γ). Histogram represents quantitation of three separate PLC-γ immunoprecipitations as described above. *B*: VSM cells were pretreated with 10 μM U-73122, a selective inhibitor of PLC, for 30 min before treatment with 10 ng/ml IL-1β for 10 min or 0.5 μM ionomycin (Iono) for 5 min. The cell lysates were then immunoblotted for active ERK1/2 (P-ERK1/2) and total ERK1/2 (ERK2). *C*: VSM cells were pretreated with 10 μM U-73122 or 10 μM U-73443, an inactive analog of U-73122, for 30 min before treatment with 10 ng/ml IL-1β for 10 min. The cell lysates were then IB for active ERK1/2 (P-ERK1/2). The graph represents quantitation of three separate experiments.

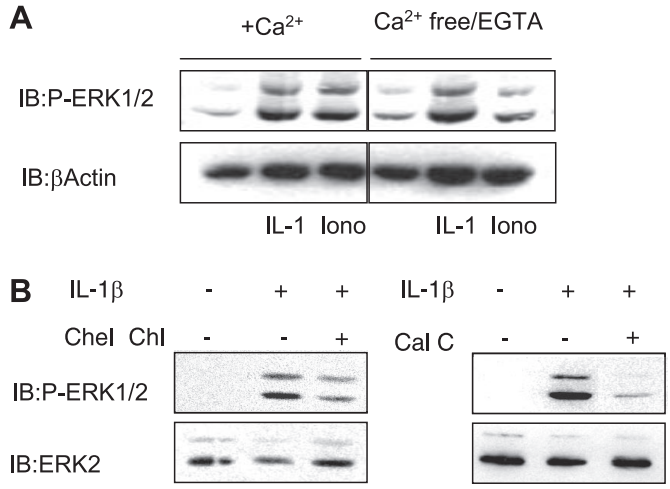

Fig. 2. Role of Ca^{2+} and protein kinase C (PKC) in IL-1β-dependent activation of ERK1/2. *A*: VSM cells were treated under standard conditions or incubated for 20 min under Ca^{2+}-free conditions with 1 mM EGTA before stimulation with 10 ng/ml Il-1β for 10 min or 0.5 μM Iono for 5 min. The cell lysates were immunoblotted for activated ERK1/2 (IB:P-ERK1/2) or β-actin (IB:β-actin) to ensure equal protein loading. *B*: VSM cells were treated with 10 μM chelerythrine chloride (Chel Chl) or 0.5 μM calphostin C (Cal C), both selective PKC inhibitors, for 30 min before a 10-min stimulation with 10 ng/ml IL-1β The cell lysates were resolved on SDS-PAGE and immunoblotted for active ERK1/2 (P-ERK1/2) and total ERK1/2 (ERK2).

kit uses a glutathione-*S*-transferase fusion protein containing the Ras-binding domain of Raf (GST-Raf-RBD) to pull down active GTP-bound Ras. The pulled-down active Ras is detected by anti-Ras immunoblot analysis.

Nitrite detection. Nitrite (NO_2^-) was detected by chemiluminescence with an NO analyzer (Sievers) as described previously (9). Briefly, 50 μl of media were injected into a reaction vessel containing acetic acid and 50 mg/ml K^+ iodide. Under these conditions, NO_2^- is reduced to NO, which is then purged out of the reaction vessel and measured by chemiluminescence. Standard curves were performed to determine media nitrite concentration, which was normalized to cell number.

Materials. Constitutively active PKC-δ and PKC-ϵ were gifts from Dr. Allan Samarel (Loyola University, Maywood, IL). Dominant-negative $p21^{ras}$ was a gift from Dr. Kevin Pumiglia (Albany Medical College). All adenovirus stocks were propagated by the addition of small amounts of virus to human embryonic kidney-293 cells. When cells were ~50% lysed, cells and media were collected, subjected to 3× freeze/thaw cycles, aliquoted, and stored at −80°C. Titer assays were performed by the method of O'Carroll et al. (34). All assays were performed using an adenovirus containing β-galactosidase as a control at matching multiplicity of infection (MOI).

Polyclonal antibodies to PKC-δ, PKC-α, and PKC-ϵ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibodies for ERK2 were purchased from Transduction Laboratories (Lexington, KY). The antibodies specific for active ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA). Antibody selective for $p21^{ras}$ was purchased from Oncogene research products. Inhibitors of PKC-δ, PKC-α, phosphatidylinositol 3-kinase, and PLC were purchased from Calbiochem (La Jolla, CA). All tissue culture media were purchased from GIBCO-BRL Life Technologies unless specifically stated. Tissue culture supplies (dishes, pipettes, etc.) were purchased from Fisher Scientific. SDS-PAGE and Western blot supplies were purchased from Bio-Rad, unless otherwise stated. All other chemicals were purchased from Sigma (St. Louis, MO).

RESULTS

Role of PLC-γ in IL-1β-dependent activation of ERK1/2. Previously, we (18) and others reported a role for ERK1/2 in mediating IL-1β-dependent expression of iNOS in VSM cells. The signaling mechanisms proximal to IL-1β stimulation, resulting in ERK1/2-dependent regulation of iNOS expression, have not been clearly elucidated. A recent study (45) in

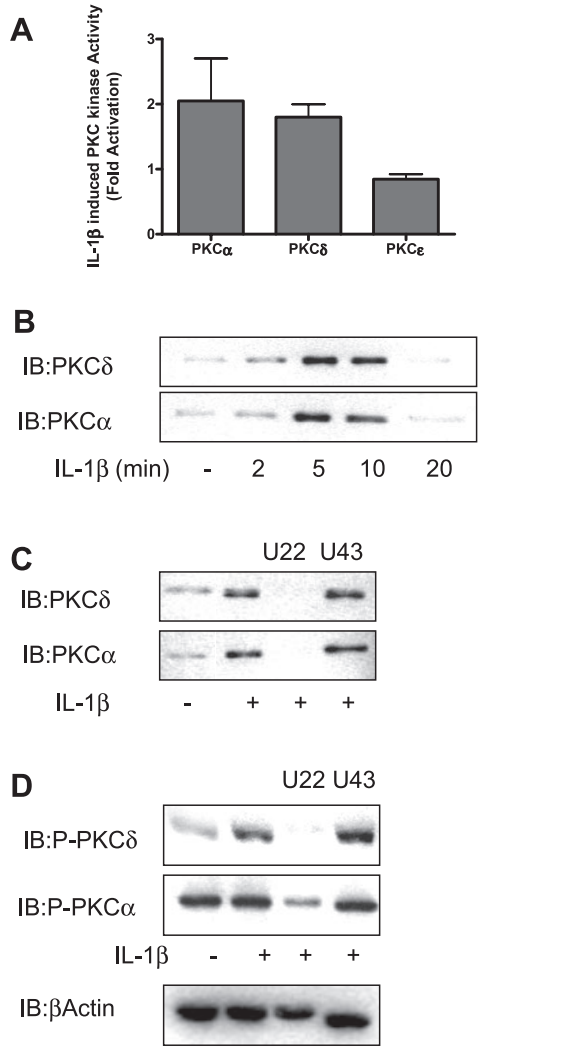

Fig. 3. IL-1β-dependent activation of PKCs in VSM cells. *A*: either PKC-α, PKC-δ, or PKC-ϵ was immunoprecipitated from VSM cells stimulated with 10 ng/ml IL-1β for 10 min. The activity of each of the PKC isozymes was determined as described in EXPERIMENTAL PROCEDURES. The graph represents the quantitation of 3 separate experiments. *B*: VSM cells were stimulated with 10 ng/ml IL-1β for the indicated times. The cells were harvested and fractionated as described in EXPERIMENTAL PROCEDURES. The soluble particulate fraction was resolved by SDS-PAGE and immunoblotted for PKC-δ (IB:PKCδ) or PKC-α (IB:PKCα). *C*: VSM cells were pretreated with 10 μM U-73122 or its inactive analog U-73443 before stimulation with 10 ng/ml IL-1β for 5 min. The cells were harvested and fractionated as described in EXPERIMENTAL PROCEDURES. The soluble particulate fraction was resolved by SDS-PAGE and immunoblotted for PKCδ (IB:PKCδ) or PKCα (IB:PKCα). *D*: VSM cells were pretreated with 10 μM U-73122 or its inactive analog U-73443 before stimulation with 10 ng/ml IL-1β. The cell lysates were then immunoblotted with antibody that specifically recognizes active PKC-δ (P-PKCδ^{ser643}) or active PKC-α (P-PKCα$^{ser638/641}$). The nitrocellulose membranes were immunoblotted with antibody for β-actin to ensure equivalent protein amounts in each lane.

gingival fibroblasts indicates a role for PLC-γ in IL-1β-induced ERK1/2 activity. In light of this evidence supporting a role for PLC-γ in IL-1β signaling, we tested whether stimulation with IL-1β would result in the activation of PLC-γ in VSM cells. Immunoprecipitations of VSM cells stimulated with IL-1β revealed tyrosine phosphorylation of PLC-γ, indicative of its activation, as early as 5 min after stimulation (Fig. 1*A*). To determine whether PLC-γ plays a role in mediating IL-β-induced ERK1/2 activation, VSM cells were treated with the PLC-selective inhibitor U-73122. Treatment with 10 μM U-73122 significantly attenuated the IL-1β-induced ERK activity but had no effect on ionomycin-stimulated ERK1/2 (Fig. 1*B*). These findings, along with our previous report (12) showing that U-73122 was ineffective in blocking phorbol 12,13-dibutyrate-dependent activation of ERK1/2, provide strong evidence that the effects of U-73122 on ERK1/2 activity can be attributed to its selective inhibition of PLC. Furthermore, treatment with comparable levels of U-73443, an inactive analog of U-73122, had little effect on IL-1β-dependent activation of ERK1/2 (Fig. 1*C*).

IL-1β activates ERK1/2 in a PKC-dependent manner in VSM cells. It is well established that activation of PLCs results in increases of intracellular [Ca^{2+}] and the production of DAG (39). Previously, we have shown that treatment of VSM cells with ionomycin (13), a Ca^{2+} ionophore that increases intracellular [Ca^{2+}], and G protein-coupled receptor agonists (11) results in activation of ERK1/2 through the Ca^{2+}/calmodulin-dependent protein kinase CaMKII. Furthermore, we have also established that activation of ERK1/2 in VSM cells is also regulated in a PKC-dependent manner (12). To determine the role of intracellular Ca^{2+} in IL-1β signaling, VSM cells were incubated under conditions previously established (44) to reduce both the extracellular and intracellular [Ca^{2+}]. These conditions had little effect on IL-1β-dependent activation of ERK1/2 while completely eliminating the ionomycin-dependent increases in ERK1/2 activity (Fig. 2*A*). To determine whether PKCs may play a role in IL-1β-dependent activation of ERK1/2, we utilized two pharmacological inhibitors of PKCs that have different mechanisms of action. Chelerythrine chloride is a noncompetitive inhibitor of PKC that targets the catalytic domain (31). Treatment with chelerythrine chloride significantly attenuated IL-1β-dependent activation of ERK1/2 (Fig. 2*A*). Calphostin C inhibits PKC activity by binding to the DAG-binding domain, thus preventing PKC association with membranes (25). IL-1β-induced ERK1/2 activity was also reduced by treatment with calphostin C (Fig. 2*B*). Taken together, these pharmacological data strongly suggest that novel PKCs are most likely involved in IL-1β-dependent activation of ERK1/2 in VSM cells.

Previously, we (11, 41) reported that the predominant PKC isozymes expressed in our VSM cultures were PKC-α and PKC-δ. Stimulation of VSM cells with IL-1β resulted in increases in PKC-α and PKC-δ kinase activity. No increase in PKC-ϵ activity was detected after treatment with IL-1β (Fig. 3*A*). Furthermore, IL-1β stimulation resulted in the translocation of both PKC-α and PKC-δ to the membrane fraction, further verifying that this cytokine treatment is capable of activating both PKC-α and PKC-δ in VSM cells (Fig. 3*B*). As described earlier, the products of PLC activation are required for activation of PKCs. Inhibition of PLC activity with

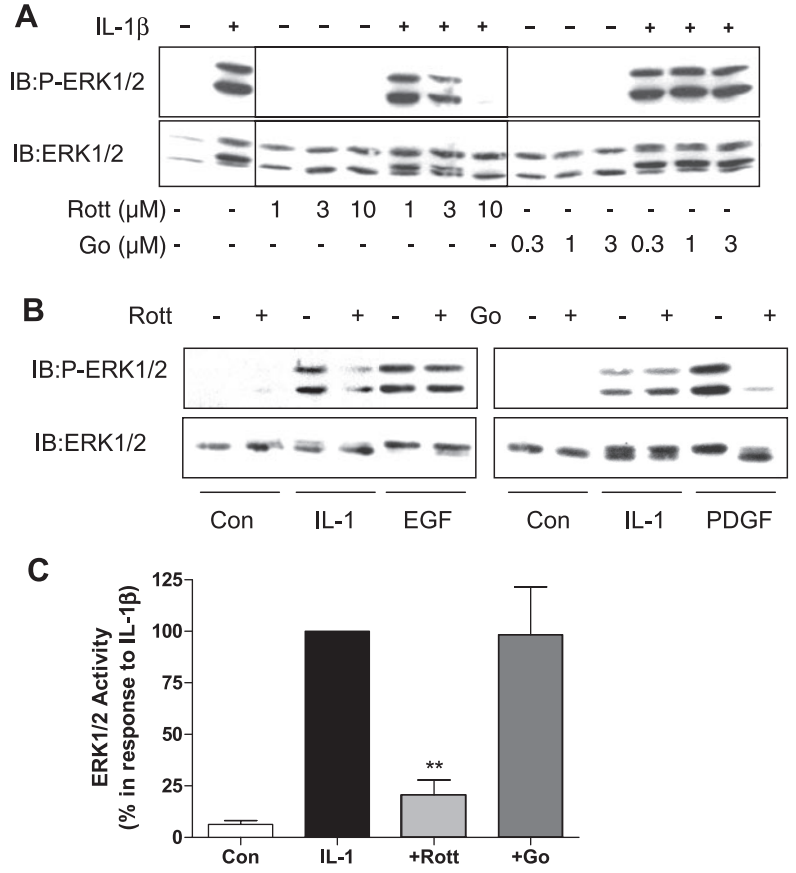

Fig. 4. PKC-δ mediates IL-1β-dependent activation of ERK1/2. *A*: VSM cells were incubated with the indicated amounts of rottlerin (Rott), a PKC-δ-selective inhibitor, or Gö-6976 (Go), a selective PKC-α inhibitor, for 30 min before a 30-min stimulation with 10 ng/ml IL-1β. The cell lysates were then immunoblotted (IB) for active ERK1/2 (P-ERK1/2) and total ERK1/2 (ERK2). *B*: VSM cells were pretreated with 3 μM rottlerin or 2 μM Gö-6976 before a 30-min stimulation with 10 ng/ml IL-1β, 5-min stimulation with 10 ng/ml epidermal growth factor (EGF), or 5-min stimulation with 10 ng/ml platelet-derived growth factor (PDGF) as indicated. ERK1/2 activation was determined as described previously. *C*: quantitation of 3 separate immunoblots using 3 μM rottlerin or 2 μM Gö-6976 to inhibit IL-1β-induced PKC activation. $^{**}P < 0.01$ as determined by ANOVA.

U-73122, the selective PLC inhibitor, prevented the IL-β-dependent translocation (Fig. 3*C*) and autophosphorylation of PKC-α and PKC-δ (Fig. 3*D*), suggesting that both of these PKC isozymes are activated in a PLC-dependent manner in response to IL-β stimulation. Previously, we (12) reported in VSM cells that PKC-δ is tyrosine phosphorylated as well as activated in response to PDGF in VSM cells. IL-1β stimulation, although capable of activating PKC-δ, does not result in PKC-δ's tyrosine phosphorylation (data not shown).

To elucidate PKC-α and PKC-δ's role in IL-1β-dependent activation of ERK1/2 we utilized the PKC isozyme-selective pharmacological inhibitors rottlerin (PKC-δ) and Gö-6976 (cPKC; PKC-α). Treatment with rottlerin inhibited IL-1β-dependent ERK1/2 activity in a concentration-dependent manner, consistent with the reported IC_{50} of rottlerin (17), whereas treatment with Gö-6976 up to 3 μM had no effect (Fig. 4*A*). Although rottlerin was effective in attenuating IL-1β-induced ERK1/2 activation, it had little effect on EGF-stimulated ERK1/2 activity, suggesting that rottlerin's ability to inhibit IL-1β-dependent ERK1/2 activity is not due to nonspecific effects of the pharmacological reagent (Fig. 4*B*). Consistent with our earlier report (12), 2 μM Gö-6976 was sufficient to attenuate PDGF-dependent activation of ERK1/2, supporting the conclusion that PKC-α is not involved in the IL-1β-dependent pathway (Fig. 4*B*).

To further verify PKC-δ involvement in IL-1β-dependent activation of ERK1/2, a complementary molecular approach was used. PKC-δ activity is regulated in part through the interaction of its pseudosubstrate domain with the catalytic domain (16, 20). It has been shown that prevention of that interaction through point mutations in the pseudosubstrate domain renders the PKC constitutively active (20). Recently, Heidkamp et al. (20) reported that expression of constitutively active PKC-δ (CaPKC-δ, A159E) in cardiomyocytes results in the activation of ERK1/2. Adenovirally overexpressed CaPKC-δ in VSM cells was autophosphorylated (Ser^{643}) independent of agonist stimulation and resulted in a dose-dependent increase of ERK1/2 activity (Fig. 5*A*). Overexpression of CaPKC at 10 MOI adenovirus increased PKC-δ kinase activity in VSM cells approximately twofold (Fig. 5*A*). While the ERK1/2 activity in the absence of IL-1β stimulation at this level of PKC-δ overexpression (10 MOI) was not significantly elevated over basal, IL-1β stimulation in conjunction with this level of CaPKC-δ overexpression resulted in significantly enhanced ERK1/2 activity over IL-1β alone (Fig. 5*C*). Importantly, equivalent overexpression of CaPKC-ϵ, a novel PKC isoform also present in VSM cells, failed to drive the IL-1β-dependent ERK1/2 activation (Fig. 5*B*), suggesting that the enhancement of IL-1β-dependent ERK1/2 activation in the

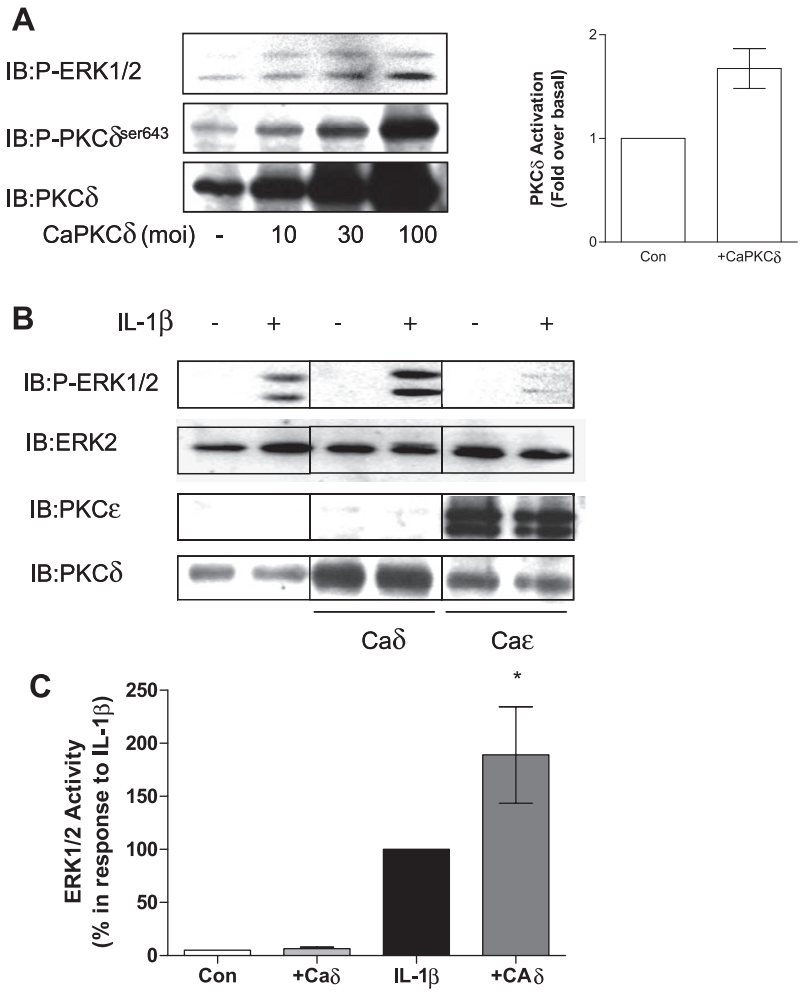

Fig. 5. Effect of adenovirus overexpression of constitutively active PKCδ on IL-1β induced ERK1/2 activity. *A*: VSM cells were infected with the indicated multiplicity of infection (MOI) of virus encoding constitutively active PKCδ (Adca-PKC-δ) for 24 h. The cell lysates were immunoblotted for active PKC-δ (IB: P-$PKC\delta^{ser643}$) (*left*). VSM cells infected with 10 MOI Ad-caPKCδ were harvested and tested for PKC-δ kinase activity, as described in EXPERIMENTAL PROCEDURES (*right*). *B*: VSM cells were infected with 10 MOI adenovirus encoding constitutively active PKC-δ (AdcaPKC-δ) or PKC-ϵ (AdcaPKC-ϵ) for 24–48 h before stimulation with 10 ng/ml IL-1β for 30 min. The cell lysates were immunoblotted for active ERK1/2 (P-ERK1/2) and total ERK1/2 (ERK2). Expression of Ca-PKC-δ (IB:PKC-δ) or Ca-PKC-ϵ (IB:PKC-) was determined by immunoblot analysis. *C*: quantitation of 3 separate experiments. $^*P < 0.05$ as determined by ANOVA.

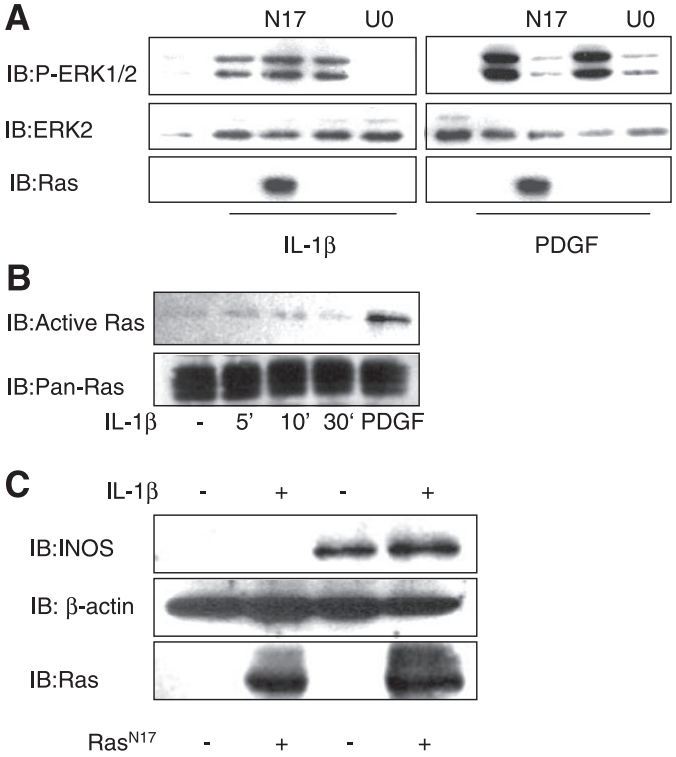

Fig. 6. Role of Ras in IL-1β-induced ERK1/2. *A*: VSM cells were infected with 20 MOI adenovirus encoding dominant-negative Ras (Ras^{N17}) for 24 h or 10 μM U0126, a selective MEK inhibitor, before stimulation with 10 ng/ml IL-1β for 30 min or 10 ng/ml PDGF for 5 min. The presence of active ERK1/2 (P-ERK1/2) was determined by immunoblotting as described earlier. Total ERK (ERK2) and expression of Ras^{N17} were also determined by immunoblot analysis. *B*: VSM cells were treated with 10 ng/ml IL-1β for the indicated times and 10 ng/ml PDGF for 5 min. The cells were harvested and assayed for Ras activity as described in the EXPERIMENTAL PROCEDURES. Activated ras (IB:Active Ras) and total Ras (IB:Pan-ras) were detected by immunoblot analysis with a pan-Ras antibody. *C*: VSM cells were infected with Ras^{N17} adenovirus as described above before stimulation with 10 ng/ml IL-1β for 24 h. Expression of inducible nitric oxide synthase (iNOS) was determined by immunoblot analysis with antibody against iNOS (IB:iNOS). The nitrocellulose was immunoblotted with antibody against β-actin (IB:β-actin) to ensure equal protein loading in each lane and immunoblotted with antibody against Ras (IB:Ras) to determine overexpression of Ras^{N17}.

presence of increased PKC-δ is due to a specific role for PKC-δ in IL-1β-dependent activation of ERK1/2.

$p21^{ras}$ and Raf1 are not required for IL-1β-dependent activation of ERK1/2. Overexpression of a dominant-negative Ras mutant (Ras^{N17}) was utilized to determine the relative contribution of Ras-dependent pathways to IL-β-dependent ERK1/2 activation. High-efficiency infection of VSM cells with Ad.HA-Ras^{N17} resulted in robust expression of the dominant-negative Ras construct after 24 h (Fig. 6*A*). The efficacy of Ras^{N17} overexpression was confirmed by complete inhibition of PDGF-stimulated ERK1/2 activation. Under these conditions, IL-1β-stimulated ERK1/2 activation was not inhibited by Ras^{N17} overexpression (Fig. 6*A*). Ras activity can be determined by the ability of Ras to interact with a portion of Raf identified as the Ras binding domain (RBD). No Ras was detected in a RBD pull-down assay, suggesting that treatment with IL-1β was not effective in activating Ras (Fig. 6*B*). Previously, we (18) established that IL-1β induced iNOS expression in an ERK1/2-dependent manner in VSM cells. The present findings are consistent with our previous findings (18), where overexpression of Ras^{N17} was not effective in attenuating IL-1β-dependent iNOS expression (Fig. 6*C*). Taken together, these data strongly suggest that IL-1β-induced ERK1/2 activation and iNOS expression occurs in a Ras-independent manner.

Raf1 is the prototypical MEK kinase and is clearly involved in the regulation of ERK1/2 under most circumstances (38). Although typically identified as downstream of Ras in ERK1/2 signaling cascades, there are reports of its ability to be activated and to mediate ERK1/2 activation in a Ras-independent manner (42). Treatment of VSM cells with the Raf inhibitor GW5074 was ineffective in blocking IL-1β-dependent ERK1/2 activation. On the other hand, GW5074 significantly attenuated PDGF-dependent activation of ERK1/2 suggesting the efficacy of the Raf inhibitor and further suggesting that the IL-1β-dependent activation of ERK1/2 is not only Ras independent but also Raf independent (Fig. 7*A*). To further elucidate this point, IL-1β treatment did not result in a measurable increase of Raf1 activity compared with PDGF treatment as determined by the extent of its phosphorylation on Ser^{338} residue (Fig. 7*B*).

PKC-δ mediates IL-1β-dependent expression of iNOS. We next wanted to determine whether PLC-γ and PKC-δ's regulation of ERK1/2 activation extended to the regulation of effector genes, such as iNOS. Consistent with their regulation of IL-1β-dependent ERK1/2, treatment with the selective PKC inhibitor chelerythrine chloride and the PLC inhibitor U-73122 attenuated IL-1β-induced iNOS expression (Fig. 8*A*). Furthermore, treatment with the PKC-δ-specific inhibitor rottlerin blocked iNOS expression in response to IL-1β stimulation (Fig. 8*B*). Accumulation of the NO decomposition product (NO_2^-) in the cell media occurs upon the intracellular expression of iNOS. Along with reduced expression of iNOS, IL-1β accumulation of nitrite in media collected from cells treated with rottlerin was also significantly reduced (Fig. 8*C*), further supporting a role for PKC-δ in IL-1β-induced iNOS expression and subsequent NO production.

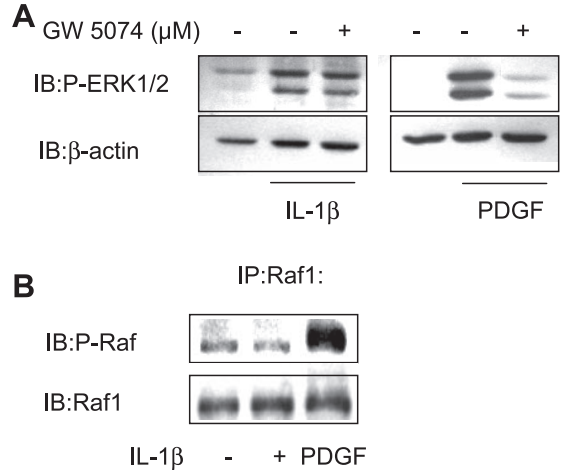

Fig. 7. Role of Raf in IL-1β-induced ERK1/2. *A*: VSM cells were pretreated with 10 μM GW5074, a selective Raf1 inhibitor, before stimulation with 10 ng/ml IL-1β or 10 ng/ml PDGF for 5 min. The cell lysates were then immunoblotted for activated ERK1/2 (IB:P-ERK1/2) and β-actin (IB:β-actin) to ensure equivalent protein loading. *B*: VSM cells were stimulated with 10 ng/ml IL-1β or 10 ng/ml PDGF for 10 min. Raf1 was immunoprecipitated from the cell lysates (IP:Raf1) and immunoblotted with antibody to activated Raf1 (IB:P-Raf1, Ser^{338}). The immunoblot was stripped and immunoblotted for total Raf1 (IB:Raf1).

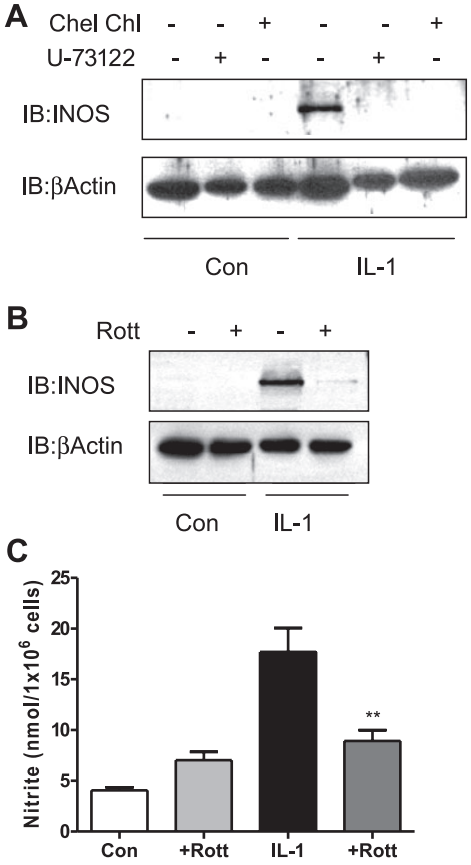

Fig. 8. PKC-δ's role in IL-1β-dependent iNOS expression. *A*: VSM cells were treated with 10 μM chelerythrine chloride or 10 μM U-73122 for 30 min before stimulation with 10 ng/ml IL-1β for 24 h. The cell lysates were then resolved on SDS-PAGE and immunoblotted with anitibody selective for iNOS (IB:iNOS). The nitrocellulose was immunoblotted with antibody selective for β-actin (IB:β-Actin) for a loading control. *B*: VSM cells were pretreated with 3 μM rottlerin (Rott) for 30 min before stimulation with 10 ng/ml IL-1β for 24 h. The cell lysates were immunoblotted with antibody against iNOS (IB:iNOS) to determine INOS expression. The nitrocellulose membrane was also immunoblotted with antibody against β-actin (IB:β-actin) to ensure equal protein amounts in each lane. *C*: expression of iNOS results in the accumulation of nitrites in the cell media. The level of nitrites in cell media from cells treated was determined as described in EXPERIMENTAL PROCEDURES. $^{**}P < 0.01$, as determined by ANOVA.

To provide additional data supporting a role for PKC-δ in regulation of IL-1β-dependent expression of iNOS and to provide an alternative to the pharmacological approaches already described, we tested the effects of adenoviral overexpressing constitutively active PKC-δ (CaPKC-δ) on IL-1β-induced iNOS. While adenoviral overexpression of CaPKC-δ did not induce expression of iNOS in the absence of IL-1β stimulation, overexpression of CaPKC-δ did result in a significant enhancement of IL-1β-induced iNOS expression (Fig. 9*A*) and NO_2^- accumulation (Fig. 9*B*). The enhancement of IL-1β-dependent iNOS expression by overexpression of CaPKC-δ was blocked by treatment with the MEK inhibitor U0126 providing strong evidence that PKC-δ's role in upregulation of iNOS is confined to its ability to regulate ERK1/2 activation (Fig. 9*C*).

DISCUSSION

Given the significant role that cytokines have in mediating the inflammatory response associated with cardiovascular disease, identifying the mechanisms that contribute to proinflammatory responses is vital. The major findings of this study are the following: *1*) PLC-γ is a proximal mediator of IL-1β-dependent activation of ERK1/2 and subsequent upregulation of iNOS in VSM cells; *2*) PKC-δ mediates this PLCγ-induced activation of ERK1/2; and *3*) this signaling pathway is independent of the classic ERK mediators Ras and Raf1.

Little is known regarding the signaling pathway(s) responsible for mediating IL-1β signaling in VSM cells. It is well established in other cell types that upon IL-1β stimulation, a series of protein-protein interactions occur, which include MyD88 association with the IL-1 receptor, followed by the recruitment of TRAF6 and IRAK (30). Although this has not been specifically shown in VSM cells, it is assumed that these are the initial, proximal events that occur. It is not clear, though, how these protein-protein interactions may lead to the activation of signaling molecules that result in increases in

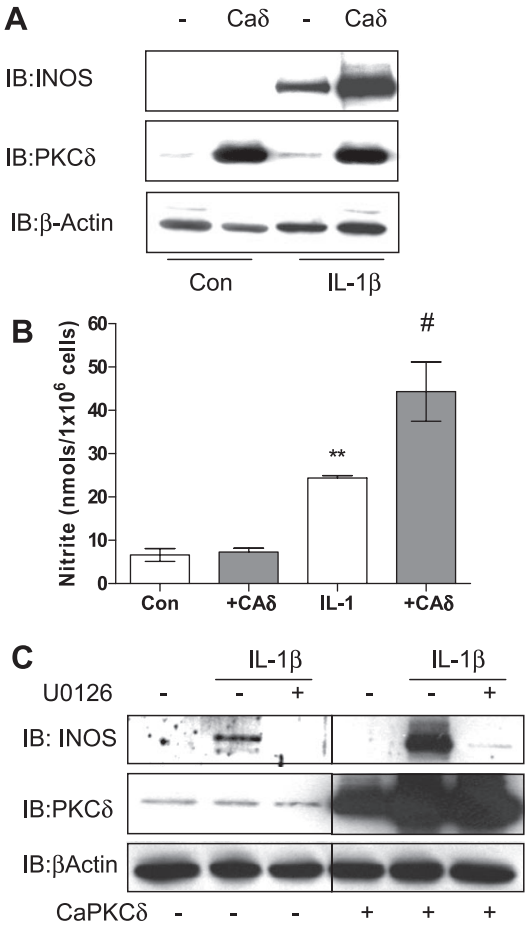

Fig. 9. Effect of adenovirus overexpression of constitutively active PKC-δ on IL-1β induced iNOS expression. *A*: VSM cells were infected with 10 MOI adenovirus encoding constitutively active PKC-δ (Caδ) for 24–48 h before stimulation with 10 ng/ml IL-1β for 24 h. Expression of iNOS was determined as described above. *B*: the level of nitrites in cell media from cells overexpressing Ca-PKC-δ treated as described above was determined as described in EXPERIMENTAL PROCEDURES. $^{**}P < 0.01$ as determined by ANOVA. $\#P < 0.05$ difference vs. IL-1β-treatment alone (also determined by ANOVA). *C*: VSM cells were infected with adenovirus encoding constitutively active PKC-δ (Caδ) as described above. Before stimulation with IL-1β for 24 h, the cells were treated with 10 μM U0126. After 24 h, the cell lysates were immunoblotted for iNOS expression (IB:iNOS), PKC-δ (IB:PKCδ), and β-actin (IB:β-Actin).

ERK1/2 activity. Recent studies (27, 28, 45) in gingival fibroblasts have demonstrated that IL-1β treatment results in a protein complex of IRAK, SHP2, and PLC-γ that is necessary for PLC-γ's activation and IL-1β-dependent ERK1/2 activation. Our study in VSM cells confirms that PLC-γ is activated in response to IL-1β and that inhibition of PLC-γ with the PLC inhibitor U-73122 prevented increases in PKC activity, ERK1/2 activity, and iNOS expression. It is not known whether PLC-γ may be part of a signaling complex containing SHP2 and IRAK in VSM cells.

A role for PKC-δ in cytokine signaling in general (40) and specifically in IL-1β-dependent upregulation of iNOS has previously been reported (4). Vallee et al. (43) reported that PKC-δ was an essential mediator of IL-1β- and TNF-α-dependent upregulation of NF-κB activity, resulting in IL-8 and ICAM-1 expression in Caco-2 cells. Similarly, PKC-δ has been implicated in cholecystokinin-8 dependent activation of NF-κB in pancreatic acinar cells (40). In the present study, we specifically attribute a role for PKC-δ in mediating ERK1/2 activation with a consequential effect on iNOS expression. Rottlerin was one of the tools used to establish a role for PKC-δ in IL-1β-dependent activation of ERK1/2. Davies et al. (8) reported that rottlerin may have effects on cellular functions apart from its role in inhibiting PKC-δ kinase activity. With the inhibition of IL-1β-induced ERK1/2 activity by chelerythine and calphostin C (Fig. 2*B*), the enhancement of IL-1β-dependent ERK1/2 activity by the overexpression of CaPKC-δ (Fig. 5*B*), and the inability of rottlerin treatment to block EGF-dependent ERK1/2 activity (Fig. 4*B*), we are confident that the rottlerin-dependent effects can be attributed to its ability to attenuate PKC-δ activity. Previously, we (18) and others (21, 23) have shown that this IL-1β-dependent activation of ERK1/2 is upstream of and is required for NF-κB activation in VSM cells. Thus our findings are consistent with the concept that PKC-δ regulates iNOS expression through transcriptional regulation of the iNOS gene itself. Carpenter et al. (4) reported that PKC-δ mediates IL-1β-dependent expression of iNOS by stabilizing the mRNA encoding iNOS, thus identifying a post-transcriptional role for PKC-δ's role in iNOS expression. Our findings that treatment with the MEK inhibitor U-0126 completely blocks IL-1β-induced ERK1/2 activity and iNOS expression precludes PKC-δ from having an obvious posttranslational role in mediating iNOS expression as has been reported in pancreatic acinar cells (4). Further substantiating this finding is that treatment with U0126 also blocked the enhanced iNOS expression induced by constitutively active PKC-δ establishing that in VSM cells, the role of PKC-δ is confined to regulation of IL-1β-dependent activation of ERK1/2.

Our results identified PKC-δ as the isoform which links ERK1/2 to the activation of the IL-1 signaling pathway. We (11, 12) have reported that regulation of ERK1/2 by G protein-dependent-coupled receptor (GPCR) agonists such as ATP and growth factors such as PDGF require the small G protein p21Ras in VSM cells. Recently, Ras was reported to be activated in response to IL-1β stimulation in a carcinoma cell line and that overexpression of Ras resulted in the activation of p38 MAPK in response to IL-1β (32). Our data indicate that Ras is most likely not involved in IL-1β-dependent activation of ERK1/2 and iNOS expression in VSM cells. PKC-δ has been linked to the activation of both Raf and MEKK1 with PKC-δ directly interacting with and modulating their activity (14, 42). As our data (Fig. 7) indicate, Raf1 does not appear to be involved in the IL-1β-dependent activation of ERK1/2 in our system. Along with the data excluding Ras, these findings further emphasize that IL-1β signaling is mechanistically distinct from the growth factor and G protein-coupled signaling pathways that have already identified in VSM cells. The MEK kinase MEKK1 was originally identified as the upstream mediator of MEK/ERK pathway, but it was later demonstrated that Raf1 was the prominent MEK kinase involved in mediating the MEK/ERK cascade (47). MEKK1 is primarily involved in activation of c-Jun kinase (46, 47), and a recent study (48) reported that PKC-δ mediates c-Jun kinase activation through association with and activation of MEKK1. With the involvement of PKC-δ in MEKK1 activation and subsequent effects on downstream signaling, MEKK1 is an attractive candidate as an intermediary in IL-1β-dependent activation of ERK1/2 in VSM cells.

These data suggest that PKC-δ may mediate IL-1β-dependent activation of ERK1/2 in VSM cells in a Ras-independent manner through regulation of a MEK kinase that has not been conclusively identified. Throughout the literature are reports of Ras-independent pathways leading to ERK1/2 activation. However, little evidence has been reported attributing any physiological relevance of Ras-independent vs. Ras-dependent activation of ERK1/2. A better understanding of the signaling molecules involved in IL-1β-induced ERK1/2 activation may not only help us identify the specific mechanisms by which IL-1β results in ERK1/2 activation in VSM but may also help us understand how cells differentially couple specific extracellular stimuli to mechanistically diverse intracellular signaling pathways.

In summary, we have identified PKC-δ as a critical component in IL-1β-dependent activation of ERK1/2 in VSM. Furthermore, we provided evidence implicating PLC-γ as a proximal mediator and additional data excluding Ras as a component of this pathway. Further studies are needed to identify the upstream signaling molecules involved in PLC-γ activation and the proteins that specifically couple PKC-δ to the MEK/ERK signaling cascade.

ACKNOWLEDGMENTS

We thank Virginia Foster for technical assistance and Wendy Hobb for assistance in preparing the manuscript.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants R01-HL-40992 and R01-HL-49426 (to H. A. Singer), and T-32-HL-07194 (to B. J. Guikema; predoctoral training grant), and National Cancer Institute Grant CA-89366 (to D. Jourd'heuil).

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